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Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection
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generated a substrate that was extended by the polymerase to a complete 50-bp duplex molecule (Fig. 4). This confirms the result shown in Fig. 2B that Rad1-Rad10 removes the 3′ single-stranded tail, and indicates that Rad1-Rad10 cleavage products contain 3′-OH groups, the required substrate for extension by DNA polymerase. Hence, Rad1-Rad10 endonuclease products are suitable substrates for a necessary subsequent step in both the SSA recombination and NER models.

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29. Rad1 and Rad10 proteins were purified as described (*15*, *28*) [L. Bardwell, H. Burtscher, W. A. Weiss, C. M. Nicolet, E. C. Friedberg, *Biochemistry* 29, 3119 (1990)]. Constituent oligonucleotides for each substrate (see Table 1) were mixed, heated to 95°C, and annealed by cooling to room temperature. Endonuclease reactions were carried out at 37°C for 40 min in 20-μl volumes containing 50 mM tris (pH 8.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, and 1 pmol of substrate DNA. Reactions were stopped, deproteinized, and analyzed by gel electrophoresis and autoradiography. The low specific activity of Rad1-Rad10 enzyme has been previously observed (*15*, *16*).
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Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection

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Nucleotide sequence information derived from DNA segments of the human and other genomes is accumulating rapidly. However, it frequently proves difficult to use such short DNA segments to identify clones in genomic libraries or fragments in blots of the whole genome or for in situ analysis of chromosomes. Oligonucleotide probes, consisting of two target-complementary segments, connected by a linker sequence, were designed. Upon recognition of the specific nucleic acid molecule the ends of the probes were joined through the action of a ligase, creating circular DNA molecules catenated to the target sequence. These probes thus provide highly specific detection with minimal background.

The application of synthetic oligonucleotides in combination with nucleic acid–specific enzymes has brought simplicity and convenience to molecular genetic analyses. There is, however, a need for methods in which oligonucleotides can be used for localized detection of single-copy gene sequences and for distinction among sequence variants in microscopic specimens. Such methods would help to bridge the analytic gap between specific gene sequences and subcellular structures. We have developed oligonucleotide probe molecules that should be useful for localized detection of specific nucleic acids. These "padlock" probes are composed of two target-complementary segments, connected by a linker that may carry detectable functions. The two ends of the linear oligonucleotide probes are brought in juxtaposition by hybridization to a target sequence. This juxtaposition allows the two probe segments to be covalently joined by the action of a DNA ligase. Because of the helical nature of DNA, circularized probes are wound around the target strand, topologically connecting probes to target molecules through catenation, in a manner similar to padlocks. The requirement for simultaneous hybridization of two different probe segments to target molecules provides for high specificity of detection in complex populations of nucleic acids (*1*). Moreover, the act of ligation permits facile distinction among similar target sequence variants as terminally mismatched probes are poor substrates for ligases (*1*, *2*). Finally, the covalent catenation of probe molecules to target sequences described here results in the formation of a hybrid that resists extreme washing conditions, serving to reduce nonspecific signals in genetic assays.

Probes useful for circularization experiments were constructed by solid phase synthesis of oligonucleotides that contained two hybridizing regions of 20 nucleotides each, connected by a 50-nucleotide-long linker segment (Fig. 1). Phosphate groups were added at the 5′ ends of the molecules as required for enzymatic ligation. Alternatively, residues of hexaethylene glycol (HEG) were incorporated in the linker segment during standard solid phase synthesis (*3*). The HEG residues served to reduce the number of synthetic steps required to span the ends of the two target-complementary segments.

Cyclizable probes were designed to detect a 40-nucleotide target sequence, represented either by an oligonucleotide molecule or by the polylinker sequence of the single-stranded form of the circular cloning vector M13 mp18. Ligation products could be separated by denaturing polyacrylamide gel electrophoresis (Fig. 2A). In the presence of the oligonucleotide target, linear probes were efficiently converted to circular molecules with a

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distinct rate of migration. Probes interacting with M13 target molecules were converted to a species catenated to and therefore migrating with the large M13 molecule during denaturing gel electrophoresis. As the probes were labeled by the addition of a radioactive phosphate group at the 5′ terminus, only ligated molecules retained their label after treatment with alkaline phosphatase. Circular oligonucleotides are insensitive to digestion with exonuclease VII, which attacks at free 5′ or 3′ ends of DNA strands (4). Depending on how the probes are labeled, phosphatases or exonucleases could be used to remove any signal arising from unreacted probes in various assays, thus reducing background (5).

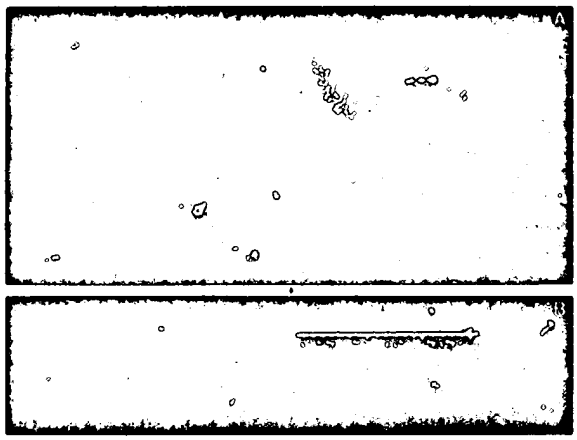

Fig. 1. Structure of a padlock probe interacting with its target sequence. (**A**) Molecular model of the probe-target complex. The molecular model was prepared on a Silicon Graphics workstation, with Insight II (Biosym Technologies). (**B**) Sequence composition of a probe, specific for a segment present in the M13 cloning vector sequence. At the 5′ end of the probe, beginning with a phosphate group, 20 target-complementary nucleotide positions are shown in red. Directly contiguous with these is a linker segment of 50 T residues, shown in green. Finally, the 20 nucleotides at the 3′ end of the probe are yellow. The target sequence is shown in blue.

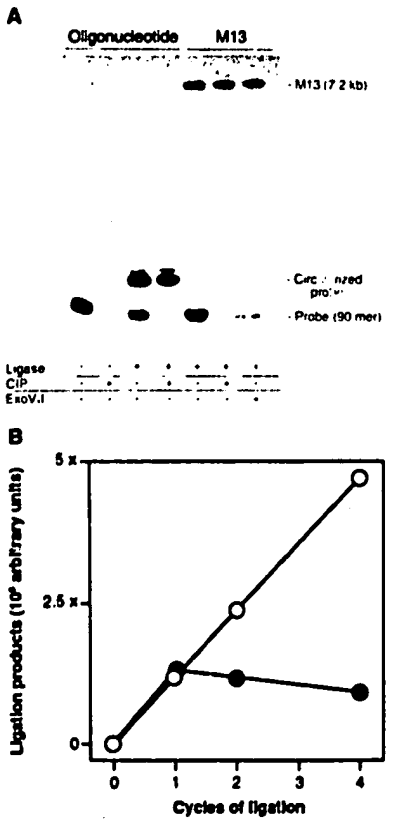

Fig. 2. Analysis by gel electrophoresis of the target-dependent circularization of an oligonucleotide probe. (**A**) A 90-bp oligonucleotide probe (5′ TGCCTGCAGGTCGACTCTAG(T)$_{50}$-CGGCCAGTGCCAAGCTTGCA-3′, see also Fig. 1B) was designed such that its 5′ and 3′ ends would hybridize adjacent to each other to a segment in the polylinker region of the M13 mp18 cloning vector. The probe was gel-purified and 5′-phosphorylated by T4 polynucleotide kinase (New England Biolabs) and γ^{32}P-ATP (3000 Ci/mmol, Dupont). To ensure that most or all 5′ ends were phosphorylated, a second kinase incubation was performed in the presence of a 20-fold excess of adenosine triphosphate (ATP). The labeled probe (6 pmol) was incubated with 3 pmol of either of two different templates: the 7.2-kb, single-stranded, circular M13 mp18 molecule or an oligonucleotide (5′-TTTTTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTTTTT-3′) that contained the same 40-bp target sequence, in 100 μl of 20 mM tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 1 mM NAD^+, 0.01% Triton X-100, and 200 U of Ampligase (Epicentre Technologies). The reactions were heated to 90°C (1 min), then cooled to 55°C (5 min) and chilled on ice. Samples (10 μl) were taken from the ligation reactions and treated with either 0.5 U of calf intestinal alkaline phosphatase (CIP; New England Biolabs) or 0.1 U of exonuclease VII (Exo VII; Gibco/BRL). (**B**) The same probe (9 pmol) was subjected to repeated cycles of ligation, separated by heat denaturation steps, in the presence of 0.3-pmol oligonucleotide target (open circles) or the circular single-stranded target molecule (filled circles). Radioactive ligation products, accumulated after the indicated number of cycles, were separated by gel electrophoresis on a 6% denaturing polyacrylamide gel and quantitated with a Phosphorimager (Molecular Dynamics).

We also investigated the consequences of cyclically repeating the probe hybridization and ligation reaction. The amount of cyclized probe molecules increased linearly with the number of ligation cycles when a short oligonucleotide target was used (Fig. 2B). By contrast, under the same conditions the maximal number of probes were bound to the closed, circular M13 target molecule in a single ligation cycle; thereafter the signal decreased, probably because of scission of the single-stranded target molecule during heat denaturation. Thus, a single probe may be catenated to each circular target molecule. This indicates that circularized probe molecules, constrained to one-dimensional diffusion along the target strand during heat denaturation, rapidly occupy the correct target sequence before new probes bind to this sequence when the temperature is lowered. Repeated cycles of ligation can, however, increase the probability that any target sequence will be detected by probe molecules specific for that target, particularly when allele-specific probes are used to distinguish among sequence variants.

Investigators can use oligonucleotide probe ligation reactions to distinguish among related DNA sequences by studying their ability to serve as templates for ligation of oligonucleotides complementary to one or the other sequence variant (*1*). Whereas probes specific for one of the two sequence variants may hybridize stably to either of the two sequences, only target molecules correctly base-paired to the juxtaposed ends of the probes can assist in the ligation. We investigated the capacity of the padlock probes to distinguish between a normal and a mutant DNA sequence in plasmid clones immobilized on nylon membranes (Fig. 3). Plasmids containing the ΔF508 variant of the cystic fibrosis transmembrane conductance regulator (CFTR) gene or the corresponding normal gene segment were spotted on nylon membranes and subjected to probe hybridization and ligation. The mutation removes 3 base pairs (bp) (6) corresponding to the 3′ end of the circularizable probe. Probe molecules specific for the normal sequence gave rise to a signal only when reacted with the normal sequence but not with the ΔF508 variant of the CFTR gene when probe ligation was followed by denaturing washes in 0.2 M NaOH for 5 min. This stringent wash (to interrupt hybridization between DNA molecules) permitted effi-

cient distinction between the allelic variants, as only cyclized probes remain bound to the membrane. By contrast, a stringent but nondenaturing wash of the same probes in a solution of 2% SDS in 0.1 × standard saline citrate (SSC) gave poor distinction between the two target sequences. Because signal strength is preserved under conditions that prevent hybridization between complementary DNA strands, nonspecifically trapped probe molecules may be efficiently removed, resulting in a reduction of the level of background in gene detection reactions.

As indicated in Fig. 2B, circularized probe molecules are free to travel considerable distances along the target strands during denaturing washes. To measure the distance traveled, probe-cyclization reactions were carried out on equivalent numbers of covalently closed target molecules or molecules that had been linearized at variable distances from the probe-complementary sequence before being immobilized on nylon membranes (Fig. 3B). Few probe molecules that were cyclized around target strands interrupted approximately 150 nucleotides from the probe-complementary sequence remained after denaturing washes. By contrast, strands digested 850 nucleotides from the probe-complement retained similar numbers of probes as did uninterrupted strands. The greater preservation of signal upon denaturing washes of probes bound to the longer linear target molecules probably reflects the increased likelihood that target molecules were cross-linked to the membrane on both sides of the site where the probe was catenated. This trapping of circularized probes by catenation to linear target molecules, in combination with the specific detection afforded by the requirement that two different probe segments simultaneously react with the target sequence, should be of value in procedures such as DNA blotting or for screening genomic libraries with short probe sequences.

Currently, oligonucleotide probes find limited applications for in situ analysis of gene sequences in metaphase chromosomes. This is a consequence of problems both with specificity of detection and sensitivity of visualization. A circularizable probe, specific for a repeated centromeric motif characteristic of human chromosome 12 (*7*), was used for in situ hybridization followed by ligation in human metaphase chromosome preparations. A wide range of washing conditions, including ones that remove specifically hybridizing oligonucleotide or longer probes preserved signals from in situ circularized probe molecules and permitted efficient distinction from alphoid repeat sequences present on other human chromosomes (Fig. 4). Given sufficiently sensitive techniques for detection of probe molecules, the high specificity of padlock probes in conjunction with the reduced nonspecific background observed should permit detection of short, single-copy DNA sequences in human chromosomes. Increased signal could be obtained by secondary ligation of detectable molecules to the linker segment of bound probes. Thus, oligonucleotide probes could be used to screen for the presence of known mutations in loci distributed along the chromosomes, by means of color coded probes specific for normal and mutant sequence

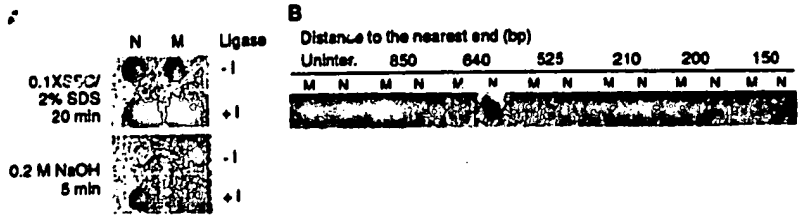

Fig. 3. Disunction of target DNA molecules immobilized on nylon membranes by means of a circularizable probe. (**A**) Fifteen femtomoles of two plasmids containing the normal or a 3-bp deleted variant of the CFTR gene were spotted on nylon membranes (PALL). The filters were treated with 0.1% SDS in boiling water and left for 10 min at room temperature; filters were then washed twice with phosphate-buffered saline (PBS) (*9*) to remove plasmids that had not been fixed to the membrane. Thirty femtomoles of a circularizable probe (5′-P TGGTGTTTCCTATGA(HEG)$_2$C-B)$_4$(HEG)$_4$AGAAATATCATCTT-3′) per microliter was hybridized to the membranes for 30 min in 5 × SSPE (*9*); 5 × Denhardt's solution (*9*), and salmon sperm DNA (500 μg/ml). The probe contained NH_2-modified C residues to which biotin had been coupled by means of a biotin-NHS ester (Clonetech Laboratories) as described (*10*). Next, the membranes were incubated for 1 hour at room temperature in a solution of 10 mM tris, pH 7.5, 10 mM $Mg(Ac)_2$, 50 mM KAc, 0.2 M NaCl, 1 mM ATP, and 0.15 U of T4 DNA ligase per microliter (Pharmacia). The membranes were washed in a solution of 2% SDS in 1 × SSPE for 30 min, next in either 2% SDS in 0.1 × SSC for 30 min for a stringent wash, or, for a denaturing wash, in 0.2 M NaOH for 5 min, and then in 1 × SSPE, 2% SDS, for 30 min. A signal was generated by incubating the membranes for 5 min in streptavidin–horseradish peroxidase conjugate (0.05 μg/ml; Boehringer Mannheim) in 2 × SSPE, 2% SDS, rinsing in PBS for 30 to 60 min, and then soaking in ECL solution (Amersham) for 1 min. The chemoluminescent signal was recorded on X-omat-S film. (**B**) Plasmids containing the normal (N) or mutant (M) variants of the target molecules were digested with restriction enzymes at the indicated distances from the sequence complementary to the probe or were left undigested. After immobilization on nylon membranes, the plasmids were probed by hybridization with a circularizable oligonucleotide, followed by a ligation step and a denaturing wash in 0.2 M NaOH.

Fig. 4. Detection of a chromosome 12–specific repeated sequence in human metaphase chromosomes, by in situ hybridization and ligation of a biotinylated circularizable probe. Metaphase chromosome preparations were obtained from a human lymphocyte culture by standard techniques of colcemide treatment, hypotonic shock, and fixation in methanol + acetic acid. In situ hybridization and ligation were performed by a modification of the procedure described (*11*). The slides were treated with ribonuclease A at 200 μg/ml in 2 × SSC (*9*) for 1 hour at 37°C, dehydrated in a series of 70, 90, 95, and 99% ice-cold ethanol washes for 2 min each, and air-dried. The chromosome preparations were then denatured in 70% formamide, 2 × SSC at 70°C for 2 min; immediately dehydrated in a series of 70, 90, 95, and 99% ice-cold ethanol washes for 2 min each; and air-dried. Circularizable probe (10 fmol/μl) specific for an alphoid repeat–motif present on chromosome 12 (5′-P AAATCTCCAACTGGAAACTG ((HEG)$_2$(C-B))$_7$(HEG)$_2$ ATTTGGTCTCAAAGTGATTG-3′) was hybridized for 18 hours at 37°C 2 × SSC, 20% formamide and salmon sperm DNA (1 μg/μl) in a 25-μl volume on each slide. A 5-min wash in 2 × SSC at 37°C and a brief wash in 10 mM tris, pH 7.5, 10 mM $Mg(Ac)_2$, 50 mM KAc, 10 mM ATP preceded ligation in the same buffer, containing T4 DNA ligase (0.085 U/μl) for 1 hour at 37°C. The slides were washed twice in 2 × SSC with 20% formamide at 37°C for 5 min each, followed by two washes in 2 × SSC and once in PN buffer (0.1 M NaH_2PO_4 0.1% NP-40, adjusted to pH 8.0 with 0.1 M Na_2HPO_4) at 37°C, 5 min each. Bound probes were visualized by means of fluorescein-labeled avidin, followed by a layer of biotinylated antibodies against avidin, both at 5 μg/ml (Vector Laboratories), and a second layer of fluoresceinated avidin. All incubations were performed in PN buffer containing 5% nonfat milk at 37°C for 20 min followed by three washes in PN buffer at room temperature for 5 min each. The metaphase chromosomes were stained with propidium iodide and photographed with a Nikon Axiofot microscope.

variants. Furthermore, probe cyclization reactions depend on an intramolecular reaction as opposed to reaction between pairs of independent probe molecules as in amplification by the polymerase chain reaction. Thus, there should be fewer problems with nonspecific reactions resulting from interactions between noncognate pairs of probe segments with cyclizable probes. The present probe design should permit the simultaneous analysis of multiple gene sequences in a DNA sample.

In conclusion, the nucleic acid probe presented here permits highly specific detection of nucleotide sequences and, although the target is not amplified, highly sensitive detection is possible through efficient reduction of nonspecific signal. Circularizable probes should be applicable in a number of other contexts, including the detection of specific RNA molecules expressed in tissue sections as T4 DNA ligase can assist in ligation reactions involving RNA strands (*8*). Moreover, immobilized padlock probes could be useful for preparative purposes, such as trapping circular target molecules from solution when screening gene libraries.

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5. The upper faint bands observed in lanes 3 and 4 probably represent small amounts of linear dimer molecules, appearing as a consequence of ligation of one end each of two different probe molecules. This material proved susceptible to exonuclease digestion. The extra lower bands in these lanes were not reproducible between experiments. Small amounts of uncatenated, circular probes appearing in lane 7 most likely were a consequence of endonuclease activity in the exonuclease preparation. With increasing amounts of exonuclease, catenated probes are lost and more free circular probes appear (M. Nilsson *et al.*, unpublished data).
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Localization of a Breast Cancer Susceptibility Gene, *BRCA2*, to Chromosome 13q12-13

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A small proportion of breast cancer, in particular those cases arising at a young age, is due to the inheritance of dominant susceptibility genes conferring a high risk of the disease. A genomic linkage search was performed with 15 high-risk breast cancer families that were unlinked to the *BRCA1* locus on chromosome 17q21. This analysis localized a second breast cancer susceptibility locus, *BRCA2*, to a 6-centimorgan interval on chromosome 13q12-13. Preliminary evidence suggests that *BRCA2* confers a high risk of breast cancer but, unlike *BRCA1*, does not confer a substantially elevated risk of ovarian cancer.

In 1990, a breast cancer susceptibility gene, known as *BRCA1*, was localized to chromosome 17q (*1*). Subsequent studies demonstrated that *BRCA1* accounts for most families with multiple cases of both early-onset breast and ovarian cancer and about 45% of families with breast cancer only, but few if any families with both male and female breast cancer (*2*). Several other genes can confer susceptibility to breast cancer. Germline mutations in the p53 gene on chromosome 17p cause a wide range of neoplasms including early-onset breast cancer, sarcomas, brain tumors, leukemias, and adrenocortical cancer (*3*). Certain rare abnormalities of the androgen receptor appear to be associated with breast cancer in men (*4*), and epidemiological studies have suggested that heterozygotes for the ataxia telangiectasia gene, *AT*, on chromosome 11q are at elevated risk of breast cancer (*5*). However, mutations in *p53* and *AT* can only be responsible for a small minority of breast cancer families that are unlinked to *BRCA1* (*6*).

To localize other genes that predispose to breast cancer, we performed a genomic linkage search using 15 families that had multiple cases of early-onset breast cancer and that were not linked to *BRCA1*. These families were classified according to the number of cases of female breast cancer, male breast cancer, and ovarian cancer (Table 1). In addition to a negative lod score (logarithm of the likelihood ratio for linkage) with markers flanking *BRCA1*, all but one of the families used for this study had at least one breast cancer case diagnosed before age 50 that did not share a *BRCA1* haplotype with other breast cancer cases in the family. The exception, CRC 136, had an obligate sporadic case diagnosed at age 53. Families were genotyped with polymorphic microsatellite repeat markers (*7*, *8*). Typing of the markers *D13S260* and *D13S263* provided provisional evidence for the presence of a susceptibility gene on chromosome 13, which was subsequently confirmed by analysis of additional polymorphisms in the region.

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